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Voak, AA; Seifert, K; Helsby, NA; WILKINSON, SR

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Evaluating aziridinyl nitrobenzamide compounds as leishmanicidal prodrugs

Andrew A. Voak[1], Karin Seifert[2], Nuala A. Helsby[3] and Shane R. Wilkinson[1].

[1]Queen Mary Pre-Clinical Drug Discovery Group, School of Biological and Chemical Sciences, Queen Mary University of London, London, UK.

[2]Immunology and Infection Department, London School of Hygiene and Tropical Medicine, London, UK

[3]Department of Molecular Medicine and Pathology, University of Auckland, Private Bag 92019, Auckland, New Zealand.

corresponding author: s.r.wilkinson@qmul.ac.uk

Running title: ***Identifying anti-parasitic nitroaromatic compounds***

Present address: Andrew Voak, Immunology and Infection Department, London School of Hygiene and Tropical Medicine, London, United Kingdom

Abstract

Many of the nitroaromatic agents used in medicine function as prodrugs and must undergo activation before exerting their toxic effects. In most cases this is catalysed by FMN-dependent type I nitroreductases (NTRs), a class of enzyme absent from higher eukaryotes but expressed by bacteria and several eukaryotic microbes including trypanosomes and *Leishmania*. Here, we utilize this difference to evaluate whether a library of aziridinyl nitrobenzamides have activity against *Leishmania major*. Biochemical screens using purified *L. major* NTR (LmNTR) revealed that compounds containing an aziridinyl-2,4-dinitrobenzyl core were effective substrates for the enzyme and showed that the 4-nitro group was important for this activity. To facilitate drug screening against intracellular amastigote parasites, we generated leishmanial cells that expressed the luciferase reporter gene and optimized a mammalian infection model in a 96-well plate format. A subset of aziridinyl-2,4-dinitrobenzyl compounds possessing a 5-amide substituent displayed significant growth inhibitory properties against the parasite, with the most potent agents generating 50 % inhibitory concentrations of <100 nM towards the intracellular form. This antimicrobial activity was shown to be LmNTR specific since *L. major* $NTR^{+/-}$ heterozygote parasites were slightly resistance to the most aziridinyl dinitrobenzyl agents tested. When the most potent leishmanicidal agents were screened against the mammalian cells in which the amastigote parasites were propagated, no growth inhibitory effect was observed at concentration up to 100 μM. We conclude that the aziridinyl nitrobenzamides represent a new lead structure that may have the potential to treat leishmanial infections.

Introduction

Leishmaniasis represents a series of insect transmitted, blood-borne diseases caused by more than 20 different protozoan parasite species belonging to the genus *Leishmania*. These infections are endemic throughout many tropical and sub-tropical countries where 350 million people are at risk of infection (1). Estimates indicate that up to 12 million individuals are currently infected by these protozoan parasites, with up to 2 million new cases and 50,000 deaths occurring each year (1). Recently, due to military activity, population migration, modern medical practices, intravenous drug usage and global warming the number of new cases in non-endemic areas has increased stimulating interest from pharmaceutical companies in these previously neglected infections (2-4). Drugs currently represent the only treatments available to combat leishmaniasis. For more than 60 years, front-line therapies have been based on pentavalent antimonial compounds but their use is problematic as they are toxic and require medical supervision to administer with clinical resistance now commonplace (5,6). In light of this worrying situation, a range of alternative treatments such as amphotericin B, paromomycin and miltefosine are now available but these too are far from ideal as they can be expensive and require medical administration, with some having teratogenic and other unwanted toxicity problems (7). Therefore, there is an urgent requirement for new, safer, cost effective anti-leishmanial treatments.

Nitroaromatic compounds are used predominantly as broad spectrum antibiotics to treat various urinary and gastrointestinal tract infections. They are characterised by possessing at least one nitro group attached to an aromatic ring, that usually has a heterocyclic structure (*e.g.* imidazole, furan or thiazole) (8). However, following concerns over their safety, the use of many nitroaromatics has been discontinued in Europe and USA although they are commonly prescribed elsewhere ((9-11); reviewed in (12)). It is now apparent that several nitro-based compounds are not as toxic as initially thought (13-15). Such observations have

stimulated renewed interested in this group of agents with calls made for the reinstatement of nitrofurantoin as a treatment for uncomplicated urinary tract infections, whilst several others have emerged as lead structures to treat various microbial infections and different forms of cancer, with fexinidazole and PA-824 undergoing evaluation to treat visceral leishmaniasis (16-24).

Most nitroaromatic compounds used in medicine function as prodrugs and must undergo activation before mediating their therapeutic effects, reactions catalysed by nitroreductases (NTRs). Based on oxygen sensitivity, flavin cofactor and the reduction products, NTRs can be broadly divided into two classes, type I or type II NTRs (25). In many bacteria and eukaryotic microorganisms, FMN-containing type I NTRs use NAD(P)H to drive the sequential two electron reduction of the conserved nitro ($-NO_2$) group to a hydroxylamine derivative (-NHOH) via an unstable nitroso intermediate (-NO) (see reactions 1 and 2). The hydroxylamine can be processed further to produce DNA damaging/crosslinking adducts and other toxic molecules such as open chain nitriles and reactive dialdehydes (18,25-32). Crucially, this reaction can occur in aerobic and anerobic environments indicating that the above reactions do not involve O_2 and as such, FMN-type I catalysed activity is said to be "O_2 insensitive". For bacteria and trypanosomes, this mechanism of activation appears to be key for the selective toxicity of many nitroaromatic prodrugs as cells selected for resistance frequently show mutations in, or decreased expression of, their FMN-type I NTR gene complement (33-35).

$$R\text{-}NO_2 + 2e^- \rightarrow R\text{-}NO \qquad (1)$$

$$R\text{-}NO + 2e^- \rightarrow R\text{-}NHOH \qquad (2)$$

In contrast, the ubiquitous NAD(P)H dependent type II NTRs use FAD or FMN as a cofactor to catalyse the one electron reduction of the conserved nitro group to form an unstable nitro anion radical ($-NO_2^{\bullet-}$). In the presence of O_2, this radical can undergo futile cycling to

produce superoxide anions (O_2^-) and regenerate the parental compound (36,37) (see reactions 3 and 4). Mammalian enzymes, such as NAD(P)H quinone oxidoreductase 1, can catalyse a two electron reduction reaction of many nitroaromatic prodrugs under aerobic conditions, however, mammalian nitroreduction does not utilise FMN containing -type I enzymes (38).

$$R\text{-}NO_2 + e^- \rightarrow R\text{-}NO_2^{\bullet -} \qquad (3)$$

$$R\text{-}NO_2^{\bullet -} + O_2 \rightarrow R\text{-}NO_2 + O_2^{\bullet -} \qquad (4)$$

Here, we exploit the activity of a FMN-type I NTR expressed by the protozoan parasite *Leishmania major* to conduct biochemical and phenotypic screens on a small library of azirindyl nitrobenzamide (ANB) compounds, agents we have previously shown to display significant anti-trypanosomal activities (39). Two of these compounds were highly active against intracellular form *L major* and displayed high selectivity toward the parasite. We postulate that compounds based around a 5-(aziridin-1-yl)-2,4-dinitrobenzamide core represent a promising new class of leishmanicidal agent.

Materials and methods

Chemicals

Aziridinyl nitrobenzamides (ANB) structures are shown in Table 1. The synthesis of NH1-8 is described elsewhere (40). CB1954 was purchased from Sigma Aldrich and NH9-12 were supplied by the Department of Therapeutics, NCI (USA).

Cell culturing.

L. major (MHOM/IL/80/Friedlin) promastigote parasites were grown at 27 ^{o}C in modified M199 medium (Life Technologies) (41). Transformed parasites were grown in this medium supplemented with G418 (20 μg mL^{-1} on agar plates, 40 μg mL^{-1} in broth) or blasticidin (10 μg mL^{-1}). *L. major* metacyclic form parasites were harvested from promastigote cultures as described (42). These were used to infect differentiated human acute monocytic leukemia (THP-1) cells at a ratio of 20 parasites per mammalian cell. The *L. major*-infected monolayers were incubated overnight at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in mammalian growth medium then washed with RPMI-1640 to remove residual parasites. *L. major* amastigote parasites were maintained in differentiated THP-1 cells at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in RPMI-1640 medium.

The human acute monocytic leukemia cell line (THP-1) was grown at 37 ^{o}C under a 5 % (v/v) CO_2 atmosphere in RPMI-1640 medium (PAA Laboratories Ltd) supplemented with 2 mM pyruvate, 2 mM sodium glutamate, 2.5 U mL^{-1} penicillin and 2.5 μg mL^{-1} streptomycin, 20 mM HEPES pH 7.4 and 10% (v/v) foetal calf serum. Differentiation of THP-1 towards macrophage-like cells was carried out using phorbol 12-myristate 13-acetate (20 ng mL^{-1}) (PMA) (Sigma-Aldrich) (43,44).

135 ***In vivo* studies.**

136 All animal experiments were conducted under licence in accordance with UK Home
137 Office regulations. *L. major* parasites were passaged through female BALB/c mice by
138 subcutaneous injection of 2×10^7 purified metacyclic parasites in 100 µL RPMI medium
139 without serum into the shaved rump. Amastigotes were harvested from skin lesions and
140 allowed to transform back to promastigotes in 5 ml M199 medium.

141

142 **Anti-proliferative assays.**

143 All assays were performed in a 96-well plate format. *L. major* promastigote parasites (5 x
144 10^5 mL^{-1}) or differentiated THP-1 cells (2.5 x 10^4 mL^{-1}) were seeded in 200 µl growth
145 medium containing different concentrations of nitroaromatic agent. After incubation at 27 ^oC
146 for 5 days (*L. major*) or at 37 ^oC for 3 days (THP-1), 2.5 µg resazurin (20 µL of 0.125 µg mL^{-1}
147 stock in phosphate buffered saline) was added to each well and the plates incubated for a
148 further 8-16 hours. Cell densities were determined by monitoring the fluorescence of each
149 culture using a Gemini Fluorescent Plate Reader (Molecular Devices (UK) Ltd, Wokingham,
150 UK) at an excitation wavelength of 530 nm, emission wavelength of 585 nm and a filter cut
151 off at 550 nm, and the drug concentration that inhibits cell growth by 50 % (IC_{50}) established.

152 Growth inhibition of luciferase expressing *L. major* amastigotes was monitored as follows.
153 THP-1 cells seeded at 2.5 x 10^4 mL^{-1} in 200 µl in growth medium containing PMA (20 ng
154 mL^{-1}) were incubated at 37 ^oC in a 5 % (v/v) CO_2 atmosphere for 3 days. Macrophage
155 monolayers were washed with mammalian growth medium then infected with purified
156 luciferase expressing *L. major* metacyclic cells (5 x 10^5 cells mL^{-1}) resuspended in 200 µL
157 mammalian growth medium. Following incubation overnight at 37 °C in a 5 % (v/v) CO_2
158 atmosphere, the cultures were washed twice in growth medium to remove non-internalised

parasites and the supernatant replaced with fresh growth medium containing the compound under investigation. Compound treated infections were incubated for a further 3 days at 37 ^{o}C under a 5 % (v/v) CO_2. The growth medium was then removed and the cells lysed in 50 µL cell culture lysis reagent (Promega). Activity was then measured using the luciferase assay system (Promega) and light emission measured on a ß-plate counter (Perkin Elmer). The luminescence is proportional to the number of live cells. The IC_{50} value for each compound was then established.

Plasmids and parasite genetic manipulation.

DNA fragments corresponding to the *L. major* 5' rRNA spacer/promoter and 3' spacer rRNA regions were amplified from *L. major* Friedlin genomic DNA and sequentially cloned into the *Trypanosoma cruzi* vector pTRIX-Luc (39) replacing the equivalent *T. cruzi* 5' rRNA spacer/promoter and 3' spacer rRNA sequences. A polypyrimidine tract/spliced leader addition site sequence located upstream the *T. cruzi MPX* gene was then isolated and inserted between the *L. major* 5' rRNA spacer/promoter region and luciferase reporter to form the integrative vector pLmRIX-Luc. Following linearization, this DNA was introduced into *L. major* promastigotes in the logarithmic phase of growth using the Human T-cell Nucleofector® kit and an Amaxa® Nucleofector™ (Lonza AG) set to program U-033.

A DNA fragment encoding for the catalytic domain of *L. major NTR* (*LmNTR*) was amplified from genomic DNA with the primers *ggatcc*CTCGACGCCGTCGAGGCCGTCG and *gaattc*CTAGAACTTGTTCCACCGCAC; lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning. The fragment was digested with *Bam*HI/*Hin*dIII then cloned into the corresponding sites of the vector pTrcHis-C (Invitrogen) to form the plasmid pTrcHisC-LmNTR.

184 **Enzyme assay.**

185 Recombinant HIS-tagged LmNTR was purified as described (45). Type I NTR activity was
186 measured spectrophotometrically by monitoring the formation of the 2- and 4-hydroxylamine
187 derivatives from the parent ANB (λ = 420 nm, ε = 1,220 M^{-1} cm^{-1}) (46). A standard reaction
188 (1 mL) containing 50 mM Tris-Cl pH7.5, 100 µM NADH and 100 µM electron acceptor was
189 incubated at room temperature for 5 min. The background reaction rate was determined and
190 the assay initiated by addition of the LmNTR (35 µg). For nifurtimox, activity was measured
191 spectrophotometrically by monitoring reduction of this compound (λ = 435 nm, ε = 19,000
192 M^{-1} cm^{-1}) (47). Enzyme activities were expressed in nmoles of NADH oxidised per minute
193 per mg LmNTR and assumes that four NADH molecules are oxidised per molecule of ANB
194 or nifurtimox reduced (46, 47).
195

196 ## Results

197 ### Construction and evaluation of luciferase expressing *Leishmania major*

198 Several drug screening systems are now in place for use with *Leishmania*, each having
199 their own advantages and disadvantages. To facilitate screening of nitroaromatic compounds,
200 which are often coloured, we developed a *L. major* line that constitutively expresses
201 luciferase. The integrative vector pLmRIX-Luc was generated by sequentially cloning DNA
202 fragments containing the 5' and 3' *L. major* Friedlin rRNA promoter/spacer sequences either
203 side of an expression cassette derived from pTEX containing the luciferase and neomycin
204 phosphotransferase genes (39). To assist luciferase mRNA processing, an untranslated
205 sequence corresponding to the *T. cruzi* mitochondrial peroxiredoxin gene polypyrimidine
206 tract/spliced leader addition site was then inserted between the 5' *L. major* rRNA region and
207 reporter gene. The DNA fragment containing *L. major* rRNA/luciferase/neomycin
208 phosphotransferase sequences was then purified following restriction digestion and
209 electroporated into promastigote form parasites. After selection with G418, the luciferase
210 activity from several clones was determined and shown to be up to 1,000-fold higher than
211 that of the parental line (Fig. 1A). The effect of luciferase expression on various *L. major* life
212 cycle stages was then evaluated. This showed that the reporter did not influence: (i) growth of
213 promastigote parasites, (ii) the ability of promastigote cells to differentiate into infective
214 metacyclic forms, (iii) invasion of tissue culture derived macrophages by metacyclics, or (iv)
215 growth of intracellular amastigote parasites (Fig. 1B and E) . Additionally the luciferase
216 expressing parasites were passaged through BALB/c mice and amastigote recovered from
217 infected animals could readily differentiate to the promastigote form and be grown in culture.
218 Therefore, it is implicit that luciferase expression has no effect on *L. major* growth,
219 differentiation, and infectivity.

A prerequisite for any drug screening assay requires that reporter levels provide an accurate representation of cell number. Using extracts derived from serially diluted promastigotes, a linear relationship between luciferase activity and parasite load was observed over the range of 625 to 160,000 cells (Fig. 1C). In the case of amastigotes such a correlation was shown by evaluating reporter levels in lysates from a fixed number of macrophages infected for 16-20 h with varying *L. major* loads (Fig. 1D): luciferase activity was linear when using a parasite:mammalian cell ratio of 2.5-80:1. With this established the luciferase expressing cells were then appraised as to whether they could be used for *in vitro* drug screens in a 96-well plate format using nifurtimox as the reference compound. For promastigote assays, cells were grown in the presence of different concentrations of the nitrofuran for 6 days and the extracts tested for luminescence activity (Fig. 2A). From the resultant dose response curves nifurtimox exhibited an IC_{50} value of 7.50 ± 0.40 μM, in line with the IC_{50} value (6.30 ± 0.10 μM) obtained when using the fluorescent vital dye resazurin. For the intracellular amastigote assays, differentiated THP-1 cells were infected with recombinant *L. major* at a ratio of 20 parasites per mammalian cell. Following 72 hours growth post-infection in the presence of drug, the luciferase activity for each culture determined from which dose response curves were generated (Fig. 2B). From these plots, the IC_{50} value for nifurtimox was calculated to be 0.58 ± 0.06 μM. When the susceptibility of differentiated THP-1 cells to nifurtimox was determined using resazurin, the IC_{50} value was calculated to be >100 μM (Table 2) demonstrating that this nitrofuran has a selective toxicity of >170 toward the intracellular parasite. Therefore, based on our data, nifurtimox shows selective killing of the *L. major* amastigotes and the observed leishmanicidal activity is not due to death of the mammalian cell.

Metabolism of nitrobenzamides by the leishmanial NTR.

Many nitroaromatic prodrugs undergo activation in reactions catalysed by FMN-type I NTRs. Here, we evaluated whether CB1954 and twelve related ANBs could function as substrates for purified HIS-tagged *L. major* NTR using NADH as an electron donor (Fig. 3). Normally, assays involving this parasite enzyme can be readily followed by monitoring the change in absorbance at 340 nm, corresponding to the oxidation of NADH. However, many ANBs undergo a considerable change in absorbance at this particular wavelength. Instead, enzyme activity was assayed by monitoring the change in absorbance at 420 nm which corresponds to the appearance of the hydroxylamine metabolite (46).

Of the thirteen compounds screened, most (ten) were metabolised by LmNTR at a rate faster than that noted for nifurtimox. These "active" compounds were related in that they all contain two nitro groups at positions 2- and 4- relative to the 1-aziridinyl ring. In contrast, the three ANBs that contain a single 2-nitro group were deemed "poor" substrates for the parasite enzyme, having negligible activity values.

Leishmanicidal activity and mammalian cytotoxicity of aziridinyl nitrobenzamides.

To determine whether there was a correlation between LmNTR activity and anti-leishmanial activity, all compounds were screened against *L. major* promastigotes and luciferase expressing amastigotes. Initial tests using a fixed concentration of compound (10 μM) were set up to evaluate the growth inhibitory properties of the ANB series. Out of the thirteen compounds tested, five had no effect on either parasite stage at this concentration (Table 2) while two others had no effect on promastigote cells but did have activity against intracellular form *L. major*. Three of the five compounds that have no effect against promastigote and amastigote parasites correspond to the ANBs previously identified as "poor" LmNTR substrates. To evaluate the growth inhibitory activities of those structures

identified by the initial screen, a series of secondary assays were performed using various concentrations of the ANB. For each agent dose response curves were drawn from which the compound's IC_{50} was determined (Table 2) (Fig. 4). Out of the six compounds tested against promastigotes, CB1954, NH10 and NH11 had IC_{50} values <1 μM. These, plus NH9, also displayed sub-micromolar IC_{50}'s against amastigote parasites with two (CB1964 and NH11) yielding values <100 nM. All compounds that display leishmanicidal activity against either of the *L. major* forms were assayed for cytotoxicity against differentiated THP-1 cells (Table 2) (Fig. 4). In all cases, the ANBs screened had no growth inhibitory effect against this mammalian line at concentrations up to 100 μM. Comparison of the mammalian cell IC_{50} with the equivalent value obtained against amastigote parasites revealed that CB1964 and NH11 displayed >2000 and >1667-fold selective toxicity toward the intracellular pathogen, respectively. This clearly demonstrates that the anti-parasitic activity displayed by these two compounds was specifically due to growth inhibition of *L. major* and not due to induction of host cell toxicity.

To demonstrate that LmNTR played a role in prodrug activiation in the parasite itself, the susceptibility of *LmNTR*$^{+/-}$ heterozygous promastigotes to CB1954, NH10 and NH11 was evaluated: attempts (16 independent transformations) to generate *L. major LmNTR*$^{-/-}$ null mutants failed to produce recombinant cells leading us to speculate that this activity is essential in insect stage parasites (48). This screening demonstrated that cells expressing lower levels of the oxidoreductase were between 2- and 4-fold more resistant to the ANB than controls: wild type *L. major* had IC_{50} values of 0.42 ± 0.01 μM, 0.49 ± 0.04 μM and 0.91 ± 0.07 μM against CB1954, NH10 and NH11, respectively, while *LmNTR*$^{+/-}$ heterozygotes displayed IC_{50} values of 1.62 ± 0.02 μM, 1.00 ± 0.07 μM and 1.77 ± 0.01 μM towards these agents.

Discussion

Several high throughput phenotypic screening approaches are now available to facilitate the search for novel drugs targeting the medically relevant, intracellular stage of *Leishmania*. Of these, the luciferase-based systems have proven to be sensitive, rapid, reproducible and versatile with tagged parasites now being used to follow the fate of the pathogen during the course of an animal model infection (49-52). Here, we constructed a luciferase expressing *L. major* line making use of an existing *T. cruzi* integration vector, modifying the expression construct such that it would integrate into the leishmanial ribosomal array (39). Characterisation of the recombinant line established that expression of the reporter had no effect on *L. major* promastigote growth, metacyclogenesis and proliferation of the intracellular amastigote form. Using luciferase expressing *L. major*, growth inhibition assays were established with nifurtimox employed as selective compound for promastigote or amastigote cells in a standardized 96-well plate format.

Nitrobenzamide-based compounds that contain an aziridinyl ring or mustard substituent have been evaluated as potential therapies targeting hypoxic cancers and the trypanosomal infections African sleeping sickness and Chagas' disease (53-55). These structures invariably function as prodrugs with their toxicity dependent on reduction of the nitro group(s) to its hydroxylamine derivatives, reactions catalysed by FMN-type I NTRs (18,21). This bioreductive trigger promotes an electronic reconfiguration on the compound's aromatic ring leading to presentation of cytotoxic moieties to the cell (55). For the treatment of hypoxic cancers, the FMN-type I NTR activity must be introduced into mammalian cells using gene- or antibody-based approaches before addition of the nitrobenzamide whereas in trypanosomes an essential endogenous enzyme can be exploited to catalyse the above nitro reduction (18,21,55). As *Leishmania* also express a FMN-type I NTR, it is postulated that

318 nitroaromatic compounds may have potential for the treatment of various forms of
319 leishmaniasis (24).

320 Following the identification that CB1954, the archetypal ANB, displayed potent anti-
321 tumor activity against the Walker 256 carcinoma, a series of derivatives have been
322 synthesised differing in the number/location of nitro groups and other substituents attached to
323 a central benzyl ring core (40,56). Using a small library (14 compounds) of these structures in
324 biochemical screens, we demonstrated that compounds having two nitro groups located at the
325 2- and 4- positions on an aromatic ring backbone relative to the aziridinyl ring were readily
326 metabolised by LmNTR. The location of an amide (or amine)-containing substituent at the 5-
327 (group Ia) or 6- (group II) position did not affect this *in vitro* activity. In contrast,
328 replacement of the 4-nitro group with an H or SO_2Me substituent (group Ib) generated
329 compounds that were not metabolized by LmNTR. These biochemical studies revealed that
330 LmNTR exhibits a slightly different substrate preference compared to *Trypanosoma brucei*
331 NTR (TbNTR). In contrast to LmNTR the trypanosomal enzyme was only able to metabolise
332 compounds having the group Ia configuration. This highlights the importance of the 4-nitro
333 group during prodrug activation with both LmNTR and TbNTR unable to metabolise
334 compounds with a group Ib arrangement (39).

335 When tested against *Leishmania*, all group Ia structures showed potent growth inhibitory
336 properties toward promastigote and/or amastigote parasites thus mirroring the biochemical
337 observations. Several of these ANBs yielded IC_{50}'s below 1 μM against the intracellular *L.*
338 *major*, including CB1954 and NH11 that had sub 100 nM values. In contrast, none of the
339 group Ib ANBs had an effect on parasite growth, consistent with observations made using the
340 trypanosomal NTRs and mammalian cells expressing *E. coli* FMN-type I NTRs, again
341 highlighting the importance of the 4-nitro group and its reduction products make in mediating
342 cytotoxicity (39,40,57). Surprisingly, of the group II compounds only NH6 displayed any

leishmanicidal activity. NH6 had a lower potency compared with its structural isomer CB1954 (CB1954 was 42- and 16-fold more effective at inhibiting amastigote and promastigote parasite growth than NH6, respectively). The decreased potency displayed by group II compounds may reflect the ability of these structures (or their reduction products) to access regions of the cell where activation (or downstream leishmanicidal processes) take place. This could be in part due to the spatial arrangement of these substituents (located at the 6- position on the benzyl ring) which may hinder the presentation of the adjacent azirindinyl cytotoxic moiety to biomolecular targets within the cellular environment.

To conclusively demonstrate the link between LmNTR and leishmanicidal activities, the susceptibility of *L. major LmNTR*$^{+/-}$ heterozygous cell line to the most effective compounds (CB1954, NH10 and NH11) was investigated (49). In this context, parasites with lower levels of NTR displayed relative resistance to all compounds tested, mirroring observations made for the trypanocidal, NTR-activated nitroaromatic prodrugs (35,39,45,47). This, in conjunction with the observation that none of the group Ia compounds displayed cytotoxicity to the macrophage-like cells within which the intracellular *L. major* were cultured, suggests that the group Ia ANBs specifically target the parasite itself rather than by promoting death to the mammalian cell.

We have now identified two leishmanidical ANB-based compounds (CB1954 and NH11) that display significant potency toward the *L. major* forms that replicate inside the mammalian host. These structures were shown to mediate their anti-parasitic activities following specific activation in a reaction catalysed by a FMN-type I NTR, an activity present in the pathogen but absent from the mammalian macrophage cells. Indeed these compounds display little or no cytotoxicity to the mammalian macrophage-like cell having >1660-fold selectivity when targeting intracellular parasites although when using *in vivo* rodent models CB1954 does exhibit hepatotoxicity, neurotoxicity and causes gastrointestinal

tract disturbances (58, 59): the toxicity/pharmacokinetics of other ANBs screened here have not been determined. Despite this group Ia compounds, particularly CB1954 and NH11, warrant further attention in the development of novel leishmanidical therapies and ideally represent one component of a new combinatorial treatment.

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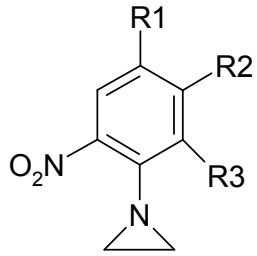
Tables

Table 1: Structure of aziridinyl nitrobenzamide compounds.



Compound	Group	Structure
CB1954	Ia	R1=NO_2; R2=$CONH_2$; R3=H
NH1	Ia	R1=NO_2; R2=$CONH(CH_2)_2$Nmorpholide; R3=H
NH2	Ia	R1=NO_2; R2=$CONH(CH_2)_2CO_2Me$; R3=H
NH9	Ia	R1= NO_2; R2=oxazole; R3=H
NH10	Ia	R1=NO_2; R2=$CONHCH_2CHCH_2$; R3=H
NH11	Ia	R1=NO_2; R2=$CONH(CH_3)CH_3$; R3=H
NH12	Ia	R1=NO_2; R2=R3=H
NH3	Ib	R1=H; R2= R2=$CONH_2$; R3=H
NH4	Ib	R1= SO_2Me; R2= $CONH_2$; R3=H
NH5	Ib	R1=SO_2Me; R2= $NHCH_2CH(OH)CH_2OH$; R3=H
NH6	II	R1= NO_2; R2=H; R3=$CONH_2$
NH7	II	R1= NO_2; R2=H; R3=$NHCH_2CH(OH)CH_2OH$
NH8	II	R1= NO_2; R2=H; R3=$CONH(CH_2)_2$Nmorpholide

571 **Table 2: Susceptibility of *L. major* and THP-1 cells to aziridinyl nitrobenzamides[a]**

compounds	group	*L. major* IC_{50} (μM)		differentiated THP-1 IC_{50} (μM)[c]	selective toxicity[d]
		promastigotes[b]	amastigotes[c]		
nifurtimox		7.50 ± 0.40	0.58 ± 0.06	>100	>172
CB1954	Ia	0.42 ± 0.01	0.05 ± 0.02	>100	>2000
NH1	Ia	7.32 ± 0.63	11.15 ± 2.01	>100	>9
NH2	Ia	1.48 ± 0.18	2.95 ± 1.14	>100	>34
NH9	Ia	>10	0.67 ± 0.05	>100	>149
NH10	Ia	0.49 ± 0.04	0.56 ± 0.05	>100	>178
NH11	Ia	0.91 ± 0.07	0.06 ± 0.01	>100	>1667
NH12	Ia	>10	1.32 ± 0.02	>100	>76
NH3-5	Ib	>10	>10	nd	nd
NH6	II	6.85 ± 0.18	2.10 ± 0.22	>100	>48
NH7-8	II	>10	>10	nd	nd

572

573 [a]nd, not determined. [b]Data are means from 4 experiments ± standard deviation. [c]Data are
574 means from 3 experiments ± standard deviation. [d]The therapeutic index of a compound was
575 calculated as a ratio of the IC_{50} against differentiated THP-1 cells to the IC_{50} against
576 amastigote parasites. Nifurtimox was used as a reference compounds.

577

Figure 1

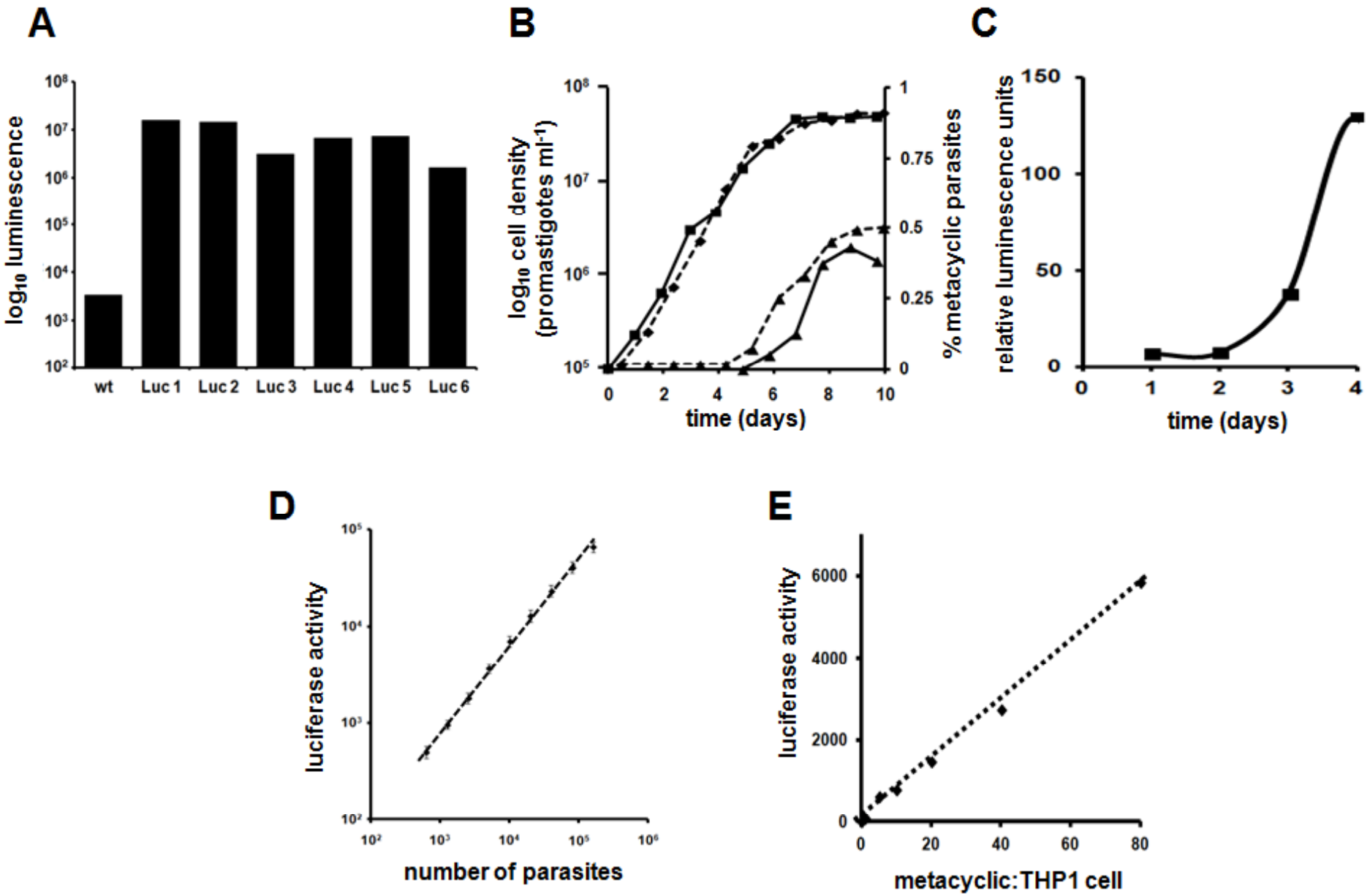

A
log10 luminescence
wt
Luc 1
Luc 2
Luc 3
Luc 4
Luc 5
Luc 6
B
log10 cell density (promastigotes ml-1)
% metacyclic parasites
time (days)
C
relative luminescence units
time (days)
D
luciferase activity
number of parasites
E
luciferase activity
metacyclic:THP1 cell

Figure 1. Luciferase expression in insect- and mammalian-stage *L. major*. (A). The luciferase activity, in relative light units, of six recombinant *L. major* promastigote clones (Luc1-6) was determined and compared to that of the parental line (wt). 1 x 10^6 cells were used in each analysis. (B). *L. major* wild type (square; solid line) and *LmNTR*$^{+/-}$*BLA* heterozygote (diamond; dotted line) promastigote parasites growth was monitored until cultures were in the stationary phase of growth. From day 5 onward, the number of metacyclic form parasites in wild type (triangle; solid line) and *LmNTR*$^{+/-}$*BLA* heterozygote (triangle; dotted line) promastigote culture was determined following purification by agglutination. The data is expressed as % metacyclics load in the total *L. major* population. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. (C). Purified *L. major* metacyclic parasites engineered to express luciferase were used to infect differentiated THP-1 cells. Over a 4 day post infection period, extracts were generated from each cell line and the luciferase activity determined. Following background correction, the luciferase activity was plotted against time. All curves shown are derived from a single data set and are representative of experiments performed in triplicate. (D). Correlation between promastigote load (between 625 to 160,000 cells) and luciferase activity. Three independent readings were taken for each parasite load and the values are means ± standard deviation. (E). Correlation between luciferase activity and *L. major* amastigote load using various parasite/macrophage cell ratios (0:1 to 80:1).

Figure 2

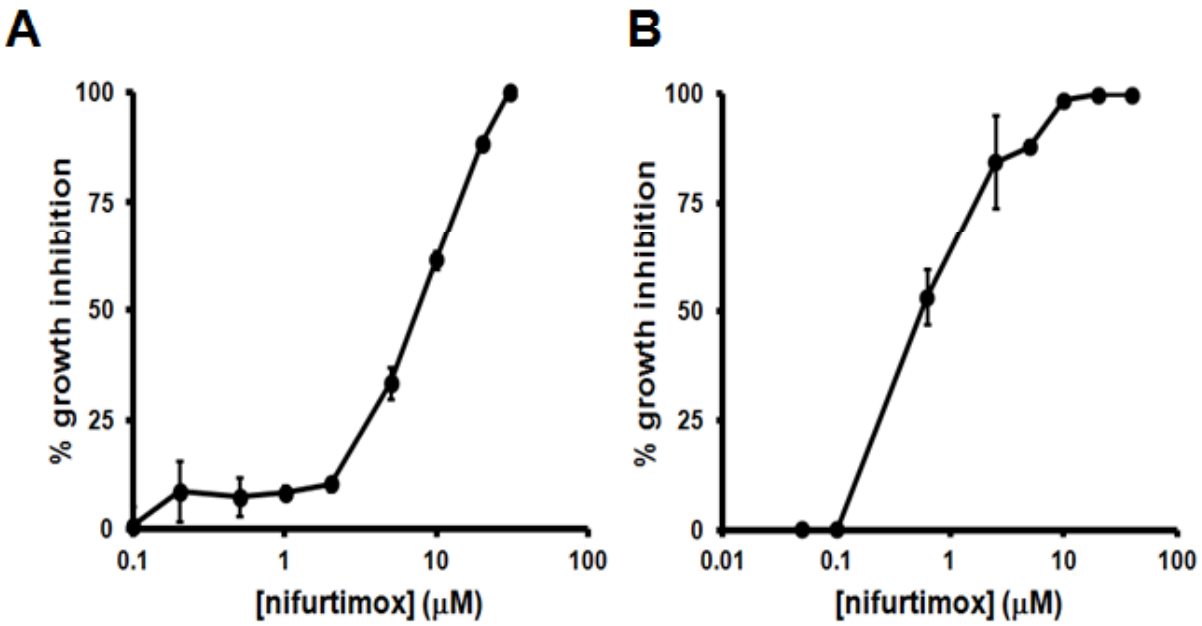


Figure 2. Susceptibility of *L. major* to nifurtimox. Dose response curves of luciferase expressing *L. major* promastigotes (A) and amastigotes (B) to nifurtimox were determined from which IC_{50} values were calculated. The data are the means from three independent experiments ± standard deviation.

Figure 3

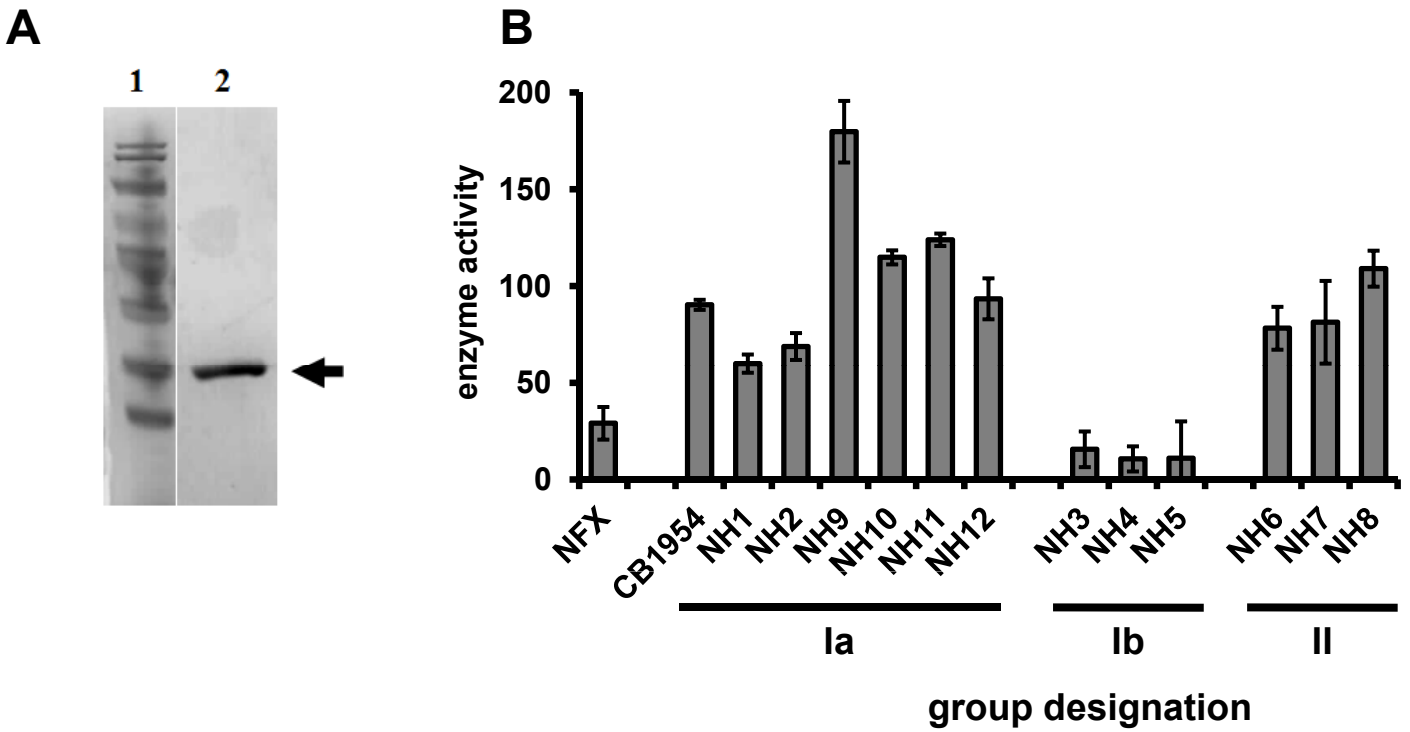


Figure 3. Activity of LmNTR toward different aziridinyl nitrobenzamides. (A) SDS-PAGE gel (10 %) stained with Coomassie blue. Lane 1, size standards; lane 2, purified HIS-tagged LmNTR. (B) The activity of purified recombinant LmNTR was assessed by using various ANBs as substrates (100 μM) at a fixed concentration of NADH (100 μM). Enzyme activity, expressed in nmoles of NADH oxidised per minute per mg LmNTR (nmol.mg^{-1}min^{-1}),, was then calculated using an ε value of 1,220 M^{-1} cm^{-1} with the assumption that four molecules of NADH are oxidised per molecule of ANB reduced (46). NFX (nifurtimox) was used as control. For nifurtimox, the change in absorbance at 435 nm was followed and enzyme activity determined using an ε value of 19,000 mM^{-1}cm^{-1}, again assuming that four molecules of NADH were oxidised per molecule of nitrofuran reduced (47). The enzyme activity values are the means of data from three assays ± standard deviations. The ANB group designations as listed in Table 1 are noted.

Figure 4

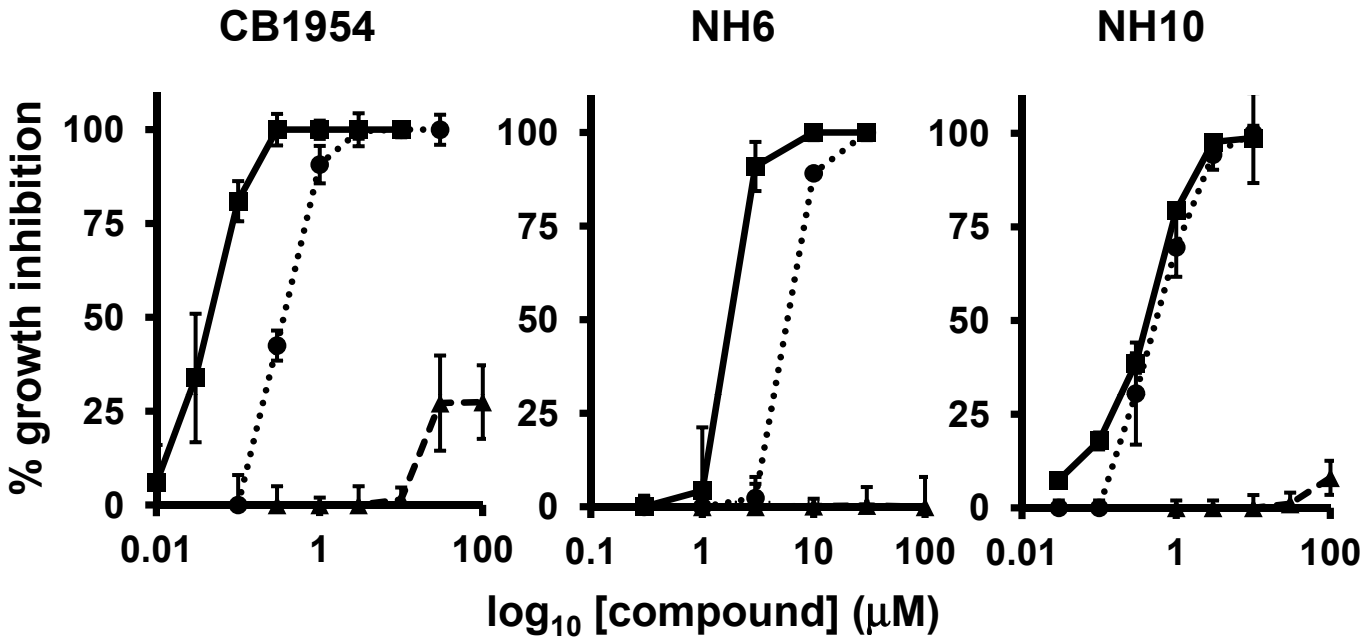


Figure 4. Dose response curves of *L. major* and mammalian cells to aziridinyl nitrobenzamides. Various concentrations of leishmanicidal ANBs were tested against *L. major* and THP-1 cells. The growth inhibitory effect of each treatment was evaluated and dose response curves constructed for promastigotes (●), amastigotes (■) and differentiated THP-1 cells (▲). In all cases, drug treatments were performed in triplicate and the plots shown represent the average growth inhibition obtained at each concentration ± standard deviation. The curves for compounds CB1954, NH6 and NH10 are shown.